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(54) Title: USE OF THE CATHELICIDIN LL-37 AND DERIVATIVES THEREOF FOR WOUND HEALING

(57) Abstract: Use of the antimicrobial cathelicidin peptide LL-37, N-terminal fragments of LL-37 or extended sequences of LL-37 having 1-3 amino acids in the C-terminal end, for stimulating proliferation of epithelial and stromal cells and thereby healing of wounds, such as chronic ulcers. The cytotoxic effect of LL-37 may be reduced by including a bilayer-forming polar lipid, especially a diacylglycerol, in pharmaceutical compositions and methods according to claim 11.

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Use of the cathelicidin LL-37 and derivatives thereof for wound healing

The present invention refers to the peptide LL-37 and N-terminal fragments, as well as functional derivatives thereof, which peptides can be used for cell proliferation, epithelial repair, and wound healing, and to a pharmaceutical composition comprising one or more of said peptides.

## BACKGROUND OF THE INVENTION

Epithelia constitute the primary barrier between host and the potentially harmful environment, and therefore the protection of this interface is vital. A wound represents a broken barrier and immediately sets in motion a series of tightly orchestrated events with the purpose to promptly reinstate the integrity of the barrier. Urgent wound closure has evolved in higher organisms, diverging from the time-consuming process of complete regeneration of tissue seen in lower species. Impaired wound healing represents a major challenge in clinical medicine ranging from the relative delay in "normal" healing seen with increasing age to pathologic non-healing ulcers.

Chronic ulcers constitute a major clinical problem and although our understanding of the physiologic wound process has increased over the past decades only minor therapeutic improvements have been attained. Distinct etiologies may underlie the development of ulcerations in different clinical conditions but, whatever the cause, non-healing ulcers are characterized by an inability of the epithelium to migrate, proliferate and close the barrier defect. The most common type of chronic skin ulcers is leg ulcers due to venous insufficiency. These patients develop peripheral venous oedema with subsequent ulceration of the skin, whereas the arterial circulation is intact. Leg and foot ulcers due to arteriosclerotic deficiencies are less common.

In addition, skin ulcers develop in association with immune diseases such as pyoderma gangrenosum and vasculitis. Current treatment includes long-term systemic immunosuppression and is not always effective. Epithelial defects and ulcers in the oral, genital and gastrointestinal mucous membranes are common and cause much distress. The underlying pathomechanisms are not always clear, such as in aphthae and erosive lichen and treatment is poor.

Traditional wound care involves removal, mechanically or enzymatically, of necrotic debris to allow formation of granulation tissue. Wounds that are heavily

colonized with bacteria may require antiseptic treatment to prevent invasive infection. Numerous topical anti-microbial agents are used, such as iodine, chlorhexidine, hydrogen peroxide, silver and antibiotics, but the risk of toxic effects of these agents on the matrix and the neoepidermis must be considered. Once the wound is clean of 5 necrotic tissue, dressings should be used to promote granulation tissue formation. A large variety of such dressings are available and numerous animal studies and clinical trials have demonstrated their beneficial effect on wound healing.

A certain proportion of wounds remain therapy-resistant and there is need for additional treatment. During the past decade there has been much focus on the 10 potential use of growth factors to accelerate wound repair. Growth factors are molecules, which control cellular processes that are critical in tissue repair, including cell migration, proliferation, angiogenesis and *de novo* synthesis of extracellular matrix. The beneficial effect of such growth factors has been suggested in a wide variety of trials (Scharffetter-Kochanek *et al.*, *Basic Res Cardiol* 93:1-3, 1999). However, to date growth 15 factor treatment of chronic ulcers has been largely disappointing in clinical practice. At present bvacaplermin (Regranex®), licensed in U.S. and Europe but not in Sweden, is the only growth factor for use, preferentially in diabetic foot ulcers. The reasons for clinical failure of growth factors in the treatment of chronic ulcers are thought to involve delivery problems and rapid degradation.

20 In parallel, there has been development of tissue therapies using autologous and allogenic materials in bioengineered human skin equivalents. Cultured epidermal keratinocytes constitute a functioning treatment for coverage of large areas of injured skin in e.g. burn patients, but is expensive, time consuming and requires laboratory facilities. To provide a dermal substrate multiple strategies have been used 25 such as acellular human cadaver and bovine collagen with or without cells. All methods available have considerable disadvantages such as potential transmission of disease and high costs and are hardly suited for basic wound care.

30 Antimicrobial peptides are effector molecules of the innate immune system, which serve to protect the host against potentially harmful microorganisms. They are conserved through evolution and are widespread in nature. In human, only a handful has been identified so far; among which the defensins and the human cathelicidin antimicrobial peptide hCAP18 have been implicated in epithelial defense (Selsted *et al.*, *J Biol Chem* 258:14485-14489, 1983).

pharmaceutical compositions containing said peptide and having an antimicrobial activity against bacteria. The peptide was named FALL-39 after the first four amino acid residues and consisted of the 39 amino acid C-terminal part of a proprotein concomitantly identified by three separate groups (Cowland *et al.*, *FEBS*, 1995; Agerberth *et al.*, 5 *Proc Natl Acad Sci USA* 1995; Lerrick *et al.*, *FEBS Letters* 1996). The peptide was shown to have potent antimicrobial activity against both gram-positive and gram-negative bacteria. Further characterization of the C-terminal peptide demonstrated a shorter sequence comprising 37 amino acids excluding the first two (FA) resulting in LL-37, which is the accepted current designation (Gudmundsson *et al.*, *Eur J Biochem* 10 238:325-332, 1996).

The proprotein was named hCAP18, human cationic anti-microbial protein, and is a member of the cathelicidin family of proteins consisting of cathelin, which has been conserved through evolution and a C-terminal part, variable in different species. In man, hCAP18 is the only member of this protein family, whereas in other species, such as mouse and pig, there are several members. The C-terminal peptide LL-37 is thought to function extracellularly and there is no evidence for intracellular cleavage of the proprotein. hCAP18/LL-37 is present in leukocytes and in barrier organs such as skin, mucous membranes, respiratory epithelium and reproductive organs. The localization of hCAP18/LL-37 to barrier epithelia seems to be consistent with a protective role for the 15 peptide in preventing local infection and systemic microbial invasion. LL-37 is described as a cysteine-free peptide that can adopt an amphiphatic, or in other words amphiphilic,  $\alpha$ -helical conformation. A high cationicity in combination with a stabilized amphiphatic  $\alpha$ -helical structure seems to be required for the anti-microbial effect of such peptides 20 against gram-positive bacteria and fungi, as has been shown experimentally (Giangaspéro *et al.*, *Eur J Biochem* 268:5589-5600, 2001). The amphiphatic and  $\alpha$ -helical structure seems to be less critical for killing of gram-negative bacteria. In association 25 with inflammation hCAP18/LL-37 is upregulated in skin epithelium (Frohm *et al.*, *J Biol Chem* 272:15258-15263, 1997) and mucous membranes (Frohm Nilsson *et al.*, *Infect Immun* 67:2561-2566, 1999).

30

#### PRIOR ART

Dorschner *et al.*, *J Invest Dermatol* 117:91-97, 2001, demonstrated that the expression of cathelicidins was increased in human and murine skin after incision, and

that lack of the murine homologue cathelicidin gene fails to protect against invasion of Group A streptococci in such mice.

WO 96/09322, Children's Medical Center Corporation, discloses that the antibacterial peptide PR-39 possesses syndecan-1 and -4 inductive activity and therefore simultaneously could reduce infection and, as a synducin, influence the action of growth factors, matrix components, and other cellular effectors involved in tissue repair. The synducins could be administered in a pharmaceutical carrier, such as conventional liposomes.

EP 0 935 965 A1, Toray Industries, Inc., refers to an antipylori agent containing an anti-microbial peptide, such as the porcine peptide PR-39, as an active agent. It is concluded that exogenous administration of PR-39 has anti-microbial activity against *Helicobacter pylori* and accelerates healing of gastric ulcers in rat. FALL39 is mentioned as one of the members of the cathelin family.

US 6,255,282, Helix Biomedix, Inc., discloses novel synthetic lytic peptides sharing structural and functional properties of different known lytic peptides. Especially a peptide of 18 to about 40 amino acids and having an  $\alpha$ -helical conformation is described. The lytic cathelicidin peptides, however, are not mentioned.

Frohm Nilsson, *Thesis*, Karolinska Institutet, Stockholm 2001, concomitantly demonstrated that human cathelicidin anti-microbial protein, hCAP18, is induced in human skin wounding, with high levels and release of active C-terminal peptide, LL-37, in physiological healing but not in chronic non-healing ulcers. hCAP18 was detected in the wound bed and in the epithelium during normal wound healing but was absent in the epithelium of chronic leg ulcers and was detected only in the wound bed and stroma. It was speculated that low levels of hCAP18 and the lack thereof in the epithelium of chronic ulcers contribute to impaired healing.

Zasloff, *Nature* 415:389-395, 2002, in a review of anti-microbial peptides discusses the diverse applications, which have been demonstrated for said peptides as anti-infective agents, and anti-microbial peptides in pharmaceutical development are described.

EP 1 358 888 A1, Bals *et al.*, having a date of publication of November 5, 2003, relates to the use of the peptide LL-37 for prevention or treatment of a disease caused by reduced blood flow and arteriosclerosis and for treatment of wounds due to reduced arterial blood supply. The ability of LL-37 to induce formation of new blood

vessels and to stimulate proliferation of endothelial cells is shown. The invention relates entirely to the angiogenetic effect and there is no mentioning of epithelia.

Although a therapeutic use of anti-microbial peptides, in particular LL-37, has been suggested, this has so far not been realized. At high concentrations of the peptide, LL-37 exerts a cytotoxic effect. The potential cytotoxic effects exerted by LL-37 are, however, inhibited in the presence of serum, but pharmaceutical formulations containing serum should be avoided due to risk for transmitting diseases, restricted accessibility and high costs.

## 10 SUMMARY OF THE INVENTION

The human anti-microbial peptide hCAP18 is up-regulated in skin epithelium as a normal response to injury. However, in chronic non-healing leg ulcers only low levels of hCAP18 were found. Notably, in the chronic leg ulcers, hCAP18 and LL-37 were entirely absent in the epithelium but present in the inflammatory infiltrate in the wound bed and in the stroma. We have now shown that hCAP18 is induced during re-epithelialization of organ-cultured skin wounds, and that this re-epithelialization was inhibited by antibodies against LL-37 in a concentration-dependant manner. These findings suggest that LL-37 plays a crucial role in wound closure, functioning as a growth factor. The invention concerns the use of LL-37 or a new synthetic peptide derived from LL-37 or a functional derivative thereof, to compensate for the lack of natural LL-37 produced *in vivo*.

It was also shown that up-regulation of hCAP18 and/or adding LL-37 peptide stimulate proliferation of normal epithelial and stromal cells, suggesting that normal wound healing and epithelial regeneration could also be enhanced.

25 It was also found that the cytotoxicity of LL-37 could be reduced in a composition comprising certain lipids.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing of the 18 kDa hCAP18 protein consisting of a signal peptide, S.P., the conserved cathelin part, and the anti-microbial peptide LL-37, which is enzymatically cut off *in vivo*.

Figure 2 is a schematic drawing of the cathelicidin protein family, illustrating the diversity of C-terminal peptides in different species.

Figures 3A, 3B, and 3C show the cDNA sequence of the pIRES2-EGFP vector including the coding sequence for hCAP18, used for transgenic expression of hCAP18.

## 5 DESCRIPTION OF THE INVENTION

The present invention refers to a peptide having a sequence of at least 20 amino acids of the N-terminal fragment of LL-37, with the proviso that LL-37 is excluded, as well as to pharmaceutically acceptable salts and derivatives thereof. LL-37 has the amino acid sequence SEQ ID NO 1:

10 H-Leu-Leu-Gly-Asp-Phe-Phe-Arg-Lys-Ser-Lys-Glu-Lys-Ile-Gly-Lys-Glu-Phe-Lys-Arg-Ile-Val-Gln-Arg-Ile-Lys-Asp-Phe-Leu-Arg-Asn-Leu-Val-Pro-Arg-Thr-Glu-Ser-OH.

The N-terminal sequence of LL-37 refers to a sequence beginning with the amino acid residue number 1 of leucine, Leu.

15 Pharmaceutically acceptable salts contain for instance the counterions acetate, carbonate, phosphate, sulphate, trifluoroacetate, and chloride. A preferred salt is the acetate. Esters and amides are examples of pharmaceutically acceptable derivatives.

20 The peptide of the invention should have an amino acid chain of no more than 40 amino acids. The invention refers to a peptide having the sequence of LL-37 to which 1-3 amino acids have been added in the C-terminal end. Any amino acid selected from Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, as well as derivatives thereof can be added. An example of a peptide having 38 amino acids, LL-38, SEQ ID NO 19, has the sequence of LL-37 to which 25 serine has been added in the C-terminal end.

The invention especially refers to a peptide having a sequence of at least 20 amino acids and selected from the group consisting of LL-36, LL-35, LL-34, LL-33, LL-32, LL-31, LL-30, LL-29, LL-28, LL-27, LL-26, LL-25, LL-24, LL-23, LL-22, LL-21 and LL-20, having the sequence SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, 30 SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, and SEQ ID NO 18, respectively.

Preferred peptides are selected from the groups consisting of LL-36, LL-35, LL-34, LL-33, LL-32, LL-31, LL-30, LL-29, LL-28, LL-27, LL-26, and LL-25.

The amino acid sequences of the peptides of the invention are given in the following table.

| SEQ ID NO | Peptide | Amino acid sequence                   |
|-----------|---------|---------------------------------------|
| 1         | LL-37   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES |
| 2         | LL-36   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES |
| 3         | LL-35   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRT   |
| 4         | LL-34   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRT   |
| 5         | LL-33   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVP     |
| 6         | LL-32   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLV      |
| 7         | LL-31   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL       |
| 8         | LL-30   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN        |
| 9         | LL-29   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLR         |
| 10        | LL-28   | LLGDFFRKSKEKIGKEFKRIVQRIKDFL          |
| 11        | LL-27   | LLGDFFRKSKEKIGKEFKRIVQRIKDF           |
| 12        | LL-26   | LLGDFFRKSKEKIGKEFKRIVQRIKD            |
| 13        | LL-25   | LLGDFFRKSKEKIGKEFKRIVQRIK             |
| 14        | LL-24   | LLGDFFRKSKEKIGKEFKRIVQRI              |
| 15        | LL-23   | LLGDFFRKSKEKIGKEFKRIVQR               |
| 16        | LL-22   | LLGDFFRKSKEKIGKEFKRIVQ                |
| 17        | LL-21   | LLGDFFRKSKEKIGKEFKRIV                 |
| 18        | LL-20   | LLGDFFRKSKEKIGKEFKR                   |
| 19        | LL-38   | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES |

5

The new peptides can be used as a medicament for cell proliferation, epithelial regeneration, healing of normal or chronic wounds, and as antimicrobial agents.

10 The new peptides are believed to have the potential to form an  $\alpha$ -helical structure at physiological conditions.

According to another aspect the invention refers to the use of a peptide having an amino acid sequence selected from the group consisting of

- 15 a) SEQ ID NO 1;
- b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;

and pharmaceutically acceptable salts or derivatives thereof, which peptide enhances proliferation of epithelial and/or stromal cells through a non-lytic mechanism, for the preparation of a medicament for epithelial regeneration, and healing of wound epithelium and stroma.

The present invention especially refers to the use of the peptide LL-37 having the amino acid sequence SEQ ID NO 1, in the form of a salt, preferably an acetate salt.

5 The invention also refers to the use of a peptide, selected from the group consisting of LL-20 to LL-36, as stated above.

LL-37, as well as LL-25 to LL-36, possesses a net positive charge (+5 - +7) at neutral pH due to the cationic amino acid residues of lysine and arginine in the primary structure. Especially LL-34 and LL-35 have net positive charge of 7. The other amino acid residues are nonpolar/hydrophobic or polar and neutral, or, to a less extent, polar 10 and negatively charged, which makes the whole peptide molecule amphiphatic. Peptides of this type interact electrostatically with the negatively charged phospholipid microbial cell walls inserting the hydrophobic face into the bilayer. A reduction of either hydrophobicity and/or charge reduces the anti-microbial effect of the peptides. The cytotoxic effect exerted by the peptides against host cells, often assessed as hemolytic 15 activity, is shown to correlate with their anti-microbial effects (Chen *et al.*, *FEBS Lett* 236:462-466, 1988). Various studies have confirmed that this is true also for other amphiphatic  $\alpha$ -helical anti-microbial peptides.

Studies of the C-terminal peptide, having a length of 37 amino acids, of rabbit CAP18 (Cap18<sub>106-142</sub>) show that broad-spectrum antibacterial activity is retained in 20 the highly basic 20 residue N-terminal sequence, but not if the N-terminus is truncated (Larrick *et al.*, *Antimicrob Agents Chemother* 37:2534-2539, 1993).

LL-37, as well as the new peptides LL-20 to LL-36, can be synthesized using an automatic peptide synthesizer and standard methods for peptide syntheses.

25 The invention especially refers to the use of the LL-37 peptide or anyone of the peptides LL-20 to LL-36 for the preparation of a medicament for treatment of chronic ulcers. Said chronic ulcers can be due to venous insufficiency, such as leg ulcers, metabolic dysfunction, such as diabetes, or immunological diseases, such as vasculites, and pyoderma gangrenosum. The peptides of the invention can also be used for treatment 30 of wounds due to trauma or burns. The described peptides can especially be used for regeneration of epithelial tissue, and to enhance epidermal regeneration following microdermabrasion.

In addition to being toxic to the cell, LL-37 is rapidly degraded in the wound environment. Serine proteinase 3 was recently shown to be responsible for extracellular cleavage of hCAP18 (Sørensen *et al.*, *Blood* 97:3951-3959, 2001).

5 In order to prevent decomposition of the peptide and also for reducing the intrinsic cytotoxicity, the peptide can be formulated with a polar lipid carrier. Said formulation should facilitate the administration of the peptide to the wound and will in addition provide a sustained release of the peptide after administration. The stability of the peptide will be improved both *in vivo* and *in vitro*.

Another object of the invention is thus a pharmaceutical composition comprising an anti-microbial cathelicidin peptide in the form of pharmaceutically acceptable salts or derivatives thereof in combination with a carrier consisting of a bilayer-forming polar lipid and an aqueous solution.

10 The cathelicidin peptide can, in addition to LL-37 in human, be derived from different animal species, and is for example SC5 from sheep, Bac5 from cow, PR-39 from pig, CRAMP from mouse, and p15 from rabbit, see Figure 2.

15 A bilayer normally refers to the lamellar arrangements of polar lipids in water. The acyl chains form the internal hydrophobic part and the polar head-groups the hydrophilic part of the bilayer. As examples of such polar bilayer-forming lipids, either of natural or synthetic origin, can be mentioned phosphatidylcholine, phosphatidylglycerol, digalactosyl-diacylglycerol, sphingomyelin and the like. Depending on the concentration of said polar lipids in polar solvents, such as water, liposomes or viscous gels of the lamellar liquid crystalline type may be formed.

20 The pharmaceutical composition especially comprises a peptide having an amino acid sequence selected from the group consisting of

- a) SEQ ID NO 1;
- b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;

25 in the form of pharmaceutically acceptable salts or derivatives thereof in combination with a carrier consisting of a bilayer-forming polar lipid and an aqueous solution.

30 Preferred bilayer-forming polar lipids to be mixed or formulated with the peptide are those, which are neutral in charge. Especially useful are the digalactosyl-diacylglycerols, and other glycolipids, such as the glycosyl ceramides, either natural or synthetic, in which a non-ionic carbohydrate moiety constitutes the polar head-group. Less preferred, but still useful, are those polar lipids, which are zwitterionic and neutral at physiological conditions, such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. Least preferred are those polar lipids, which are negatively charged and thus form strong complexes with the positively charged peptide.

According to the invention said bilayer-forming polar lipid carrier is preferably selected from the group consisting of phospholipids, galactolipids and sphingolipids.

An especially preferred bilayer-forming polar lipid is digalactosyldiacyl-glycerol or polar lipid mixtures rich in digalactosyldiacylglycerols due to the extremely good cutaneous tolerability of this class of polar lipids. Digalactosyldiacylglycerol is a class of lipids belonging to the glycolipid family, well known constituents of plant cell membranes. One of the most abundant classes contains two galactose units, and the commonly used nomenclature and abbreviation of this is digalactosyldiacylglycerol, 5 DGDG, sometimes referred to as galactolipids. Galactolipids, primarily DGDG and DGDG-rich materials have been investigated and found to be surface active material of interest in industrial applications such as food, cosmetics, and pharmaceutical products. WO 95/20944 describes the use of DGDG-rich material, a "galactolipid material", as a 10 bilayer-forming material in polar solvents for pharmaceutical, nutritional and cosmetic use. Said application does not disclose the use of galactolipids in combination with 15 peptides and proteins in general, particularly not a peptide of the present invention.

According to a preferred aspect the invention refers to a pharmaceutical composition wherein the bilayer-forming polar lipid carrier is a polar lipid mixture rich in digalactosyldiacylglycerols.

Another preferred aspect of the invention is a pharmaceutical composition 20 wherein the peptide is in the form of acetate. A preferred peptide is LL-37 in the form of an acetate salt. Especially preferred is a pharmaceutical composition comprising a combination of an acetate of LL-37 and CPL-Galactolipid as the bilayer-forming lipid carrier. CPL-Galactolipid is a trademark for a galactolipid fraction consisting of 50-70 % 25 by weight of digalactosyldiacylglycerols and 30-50 % of other polar lipids.

The ratio between the peptide in the form of a salt and a galactolipid carrier in the pharmaceutical composition should preferably be 1:5 to 1:50, especially 1:10 – 1:25 by weight.

In addition to the bilayer-forming lipid the carrier also contains an aqueous 30 solution. An aqueous solution refers to a solution having physiologically or pharmaceutically acceptable properties regarding pH, ionic strength, isotonicity etc. As examples can be mentioned isotonic solutions of water and other biocompatible solvents, aqueous solutions, such as saline and glucose solutions, and hydrogel-forming materials. The aqueous solution can be buffered, such as phosphate-buffered saline, PBS.

The pharmaceutical composition can in addition comprise pharmaceutically acceptable excipients, such as a preservative to prevent microbial growth in the composition, antioxidants, isotonicity agents, colouring agents and the like. In aqueous suspensions the compositions can be combined with suspending and stabilising agents.

5 The colloidal nature of the composition makes it possible to prepare the composition aseptically by using a final sterile filtration step.

In order to form a gel the peptide can be preferably formulated with a hydrogel-forming material. Examples of hydrogel-forming materials are synthetic polymers, such as polyvinylalcohol, polyvinylpyrrolidone, polyacrylic acid, polyethylene glycol, poloxamer block copolymers and the like; semi-synthetic polymers, such as 10 cellulose ethers, including carboxymethylcellulose, hydroxyethylcellulose, hydroxy-propylcellulose, methylcellulose, methylhydroxypropylcellulose and ethylhydroxyethylcellulose, and the like; natural gums, such as acacia, carragenan, chitosan, pectin, starch, xanthan gum and the like.

15 It is advantageous to use a hydrogel which is muco-adhesive. In that respect it is particularly useful to use hyaluronic acid and derivatives thereof, cross-linked polyacrylic acids of the carbomer and polycarbophil types, polymers that readily form gels, which are known to adhere strongly to mucous membranes.

It is also advantageous to use block copolymers of the poloxamer type, i.e. 20 polymers consisting of polyethylene glycol and polypropylene glycol blocks. Certain poloxamers dispersed in water are thermoreversible: at room temperature they are low viscous but exhibit a marked viscosity increase at elevated temperatures, resulting in a gel formation at body temperature. Thereby the contact time of a pharmaceutical formulation administered to the relatively warm wound may be prolonged and thus the 25 efficacy of the incorporated peptide may be improved.

The pharmaceutical composition of the invention can be formulated for topical or enteral, that is oral, buccal, sublingual, mucosal, nasal, bronchial, rectal, and vaginal administration.

Non-limiting examples of pharmaceutical compositions for topical administration are solutions, sprays, suspensions, emulsions, gels, and membranes. If 30 desired, a bandage or a band aid or plaster can be used, to which the pharmaceutical composition has been added. Tablets, capsules, solutions or suspensions can be used for enteral administration.

According to another aspect the invention refers to the use of a peptide having an amino acid sequence selected from the group consisting of

- a) SEQ ID NO 1;
- b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;

in the form of pharmaceutically acceptable salts or derivatives thereof for proliferation of epithelial and/or stromal cells *in vitro* through a non-lytic mechanism.

Said proliferation can especially be used for proliferation of human autologous epithelial and stromal cells *in vitro*.

The invention also refers to a growth medium for culturing eukaryotic cells, such as epithelial and/or stromal cells, which comprises LL-37 or a peptide as described in combination with a basal medium. A cytotoxicity reducing agent can be added, such as serum. Apolipoprotein A-I (apoA-I) has been found to be the main LL-37 binding protein in human plasma and works as a scavenger of LL-37 (Wang *et al*, *J Biol Chem* 273:33115-33118, 1998; Sørensen *et al*, *J Biol Chem* 274:22445-22451, 1999), suggesting a mechanism involved in the regulation of a cathelicidin peptide. The cytotoxicity reducing agent can also be a bilayer-forming polar lipid, such as a lipid selected from the group consisting of phospholipids, galactolipids and sphingolipids, as described above.

The basal medium of the growth medium of the invention is based on double-distilled water, and a number of the following ingredients: inorganic salts, phenol red, glucose, thymidine, hypoxanthine, HEPES, sodium pyruvate, aminopterin, amino acids and vitamins. For culturing of epithelial cells, such as e.g. keratinocytes, *in vitro* the growth medium can consist of basal medium and a growth promoting kit including a) LL-37 peptide in a salt solution, b) penicillin + streptomycin, c) insulin, d) transferrin, e) triiodothyronine, f) hydrocortisone, g) cholera toxin, and a selected cytotoxicity reducing agent, such as serum or a polar lipid. For culturing of stromal cells, such as e.g. fibroblasts, *in vitro*, a growth medium can consist of basal medium and a growth promoting kit including a) LL-37 peptide in a salt solution, b) penicillin + streptomycin, and a selected cytotoxicity reducing agent, such as serum or a polar lipid.

Another object of the invention is a method of enhancing the expansion of human autologous epithelial and stromal cells *in vitro* for cell transplantation *in vivo*, wherein cells are isolated from an excised piece of healthy skin, said isolated cells are cultivated *in vitro* in a growth medium according to the invention, and the cultivated cells

are subsequently harvested and used for treatment of wounds, such as burn injuries and ulcers.

The invention also refers to a growth promoting kit comprising the LL-37 peptide or a peptide as described, and a cytotoxicity reducing bilayer-forming polar lipid, 5 optionally in combination with antibiotics, basal media, and other conventional additives in separate containers.

According to still another aspect the invention refers to transfection of a full-length hCAP18 cDNA construct into autologous human keratinocytes for cell transplantation of ulcers and burns. The cDNA construct is designed to allow regulation of 10 hCAP18 gene expression by a switch mechanism (Resnitzky *et al.*, *Mol Cell Biol* 14:1669-1679, 1994). Autologous human keratinocytes are obtained from a healthy skin piece excised from the patient. The keratinocytes are isolated and expanded in cell culture as described. The cDNA construct is transfected into keratinocytes. The transfected keratinocytes are further expanded *in vitro* and given back to the patient.

15 The invention especially refers to the use of a gene construct comprising the complete cDNA sequence of hCAP18 having the sequence SEQ ID NO 20 for transfection of epithelial and/or stromal cells in order to enhance proliferation of said cells.

## 20 EXAMPLES

### Example 1. Preparation of synthetic peptides

The LL-37 peptide was synthesized according to solid phase synthesis with the 9-fluorenylmethoxycarbonyl / tert-butyl strategy. The crude peptide, as the trifluoroacetate salt, was purified with HPLC and finally isolated by lyophilization (lot 971/26, 25 from PolyPeptide Laboratories A/S, Hillerød, Denmark). The purity was determined by means of HPLC and area integration and was found to be 99 %. The molecular weight was analyzed using mass spectrometry and corresponded to the theoretical value of 4493 g/mol as the free base. Analysis of composition of amino acids showed that the relative amounts of each amino acid corresponded with the theoretical values for LL-37. 30 The peptide content was calculated from the results from the amino acid analysis and found to be 73 %, the remainder being counterions and residual solvent.

Several batches of LL-37 were synthesized, and the LL-37 peptide used in the following Examples 2 and 5 was in the form of the acetate salt.

The peptides LL-36 and LL-38 were synthesized correspondingly, in the form of acetate.

The different peptides used in the following examples and tests were as follows.

| Peptide | Counter-ion       | Lot      | Purity area-% | Peptide content % (w/w) | Used in                  | Manufact. Year |
|---------|-------------------|----------|---------------|-------------------------|--------------------------|----------------|
| LL-37   | Trifluoro-acetate | YS 5253  | 98            |                         | Ex. 3, 4, 6, 7<br>Test 3 | 1997           |
| LL-37   | Trifluoro-acetate | 971/26   | 99            | 73                      | Test 5                   | 2002           |
| LL-37   | Acetate           | 990/37/A | 99            | 83                      | Ex. 2, 5<br>Test 4       | 2003           |
| LL-38   | Acetate           | 990/38   |               |                         | Test 4                   | 2003           |
| LL-36   | Acetate           | 990/39   |               |                         | Test 4                   | 2003           |

5

Example 2. Preparation of a pharmaceutical composition comprising a mixture of LL-37 peptide and lipid carrier

A pharmaceutical composition was prepared using the following 10 ingredients:

| Ingredient                      | Concentration |
|---------------------------------|---------------|
| LL-37                           | 100 ppm*      |
| CPL-Galactolipid                | 0.20 %        |
| 2.6 % Glycerol in sterile water | ad 100 %      |

\*ppm = parts per million (by weight)

The peptide LL-37, as the acetate salt (lot 990/37/A), and the lipid carrier, CPL-15 Galactolipid, obtained from Lipocore Holding AB, a lipid material rich in digalactosyl-diacylglycerols and prepared from oats, were weighed in a 50 ml glass flask. The two ingredients were gently mixed and then the glycerol solution was added. The mixture was shaken vigorously for 120 min and then allowed to stand for 1 h. The resulting composition was a fine, homogenous dispersion. It was kept refrigerated until use.

20

Example 3. Preparation of aqueous mixtures comprising the LL-37 peptide and a lipid carrier

Mixtures of LL-37, as the trifluoroacetate salt (lot 971/26) and a polar, bilayer-forming lipid carrier were prepared using the following ingredients (percentages in weight by weight):

Table 1.

| Ingredient        | A1       | A2       | B1       | B2       | C1       | C2       |
|-------------------|----------|----------|----------|----------|----------|----------|
| LL-37             | 100 ppm  | -        | 90       | -        | 92       | -        |
| CPL-Galactolipid  | 0.19 %   | 0.20 %   | -        | -        | -        | -        |
| Epikuron 200      | -        | -        | 0.19 %   | 0.19 %   | -        | -        |
| CPL-Sphingomyelin | -        | -        | -        | -        | 0.19 %   | 0.19 %   |
| DMEM              | ad 100 % |

CPL-Galactolipid, obtained from Lipocore Holding AB, is a chromatographically purified galactolipid fraction from oats, Epikuron 200, obtained from Lucas Meyer GmbH, is phosphatidylcholine from soybean, and CPL-Sphingomyelin, obtained from Lipocore Holding AB, is chromatographically purified sphingomyelin from bovine milk. DMEM, Dulbecco's Modified Eagle Medium, from Invitrogen Corp. is an aqueous solution containing inorganic salts, glucose, phenol red, amino acids and vitamins.

The peptide LL-37 and the lipid carrier were weighed in a glass flask and then DMEM was added. The resulting dispersions were vigorously shaken, using a Heidolph Promax mixer at a frequency of 200/min for 1.5 h, and allowed to equilibrate and settle for about 3 h at room temperature. A visual assessment was then made and the following results were obtained: All samples were turbid dispersions and there were no differences in turbidity between any of the samples B1, B2, C1, and C2. The only observed difference was between samples A1 and A2: the former, containing the peptide, was significantly less turbid than the latter, without the peptide. Sample A2 was slightly less turbid than, in turn, samples B1, B2, C1, and C2. These observations indicate a stronger interaction between the two components in sample A1, which results in a smaller average particle size of the dispersion, compared to the peptide-free sample A2, but also compared to the rest of the corresponding samples. After one day of storage at room temperature samples A1 and A2 were unchanged, i.e. both were

homogeneous dispersions and A1 less turbid than A2, whereas the four other samples had considerable sediments on the bottom of the glass flasks.

All three mixtures of peptide and polar lipid carrier are useful for various purposes, e.g. as delivery systems and for tests in cell cultures; however, since the shelf-life of the mixtures of peptide and galactolipid is considerably longer (no sedimentation) than that of the others, said mixtures are the most preferred for practical use.

Example 4. Preparation of aqueous mixtures comprising a mixture of LL-37 peptide and lipid carrier

Samples of LL-37 as trifluoroacetate (lot 971/26) and a polar, bilayer-forming lipid carrier were prepared using the following ingredients (percentages in weight by weight):

Table 2.

| Ingredient             | Sample D | Sample E | Sample F | Sample G | Sample H | Sample I | Sample J |
|------------------------|----------|----------|----------|----------|----------|----------|----------|
| LL-37                  | 96 ppm   | 100 ppm  | 100 ppm  | 103 ppm  | 100 ppm  | 100 ppm  | 100 ppm  |
| CPL-Galactolipid       | 0.21 %   | -        | -        | -        | 0.20 %   | -        | -        |
| PC from soybean, 40 %  | -        | 0.21 %   | -        | -        | -        | -        | -        |
| PC from egg yolk, 60 % | -        | -        | 0.21 %   | -        | -        | -        | -        |
| DOPC, 99 %             | -        | -        | -        | 0.20 %   | -        | -        | -        |
| PC from soybean 70 %   | -        | -        | -        | -        | -        | 0.20 %   | -        |
| PC from soybean, 94 %  | -        | -        | -        | -        | -        |          | 0.20 %   |
| PBS                    | ad 100 % |

CPL-Galactolipid, manufactured by LTP Lipid Technologies Provider AB, is a chromatographically purified galactolipid fraction from oats. The various phospholipids used were phosphatidylcholine (PC) from soybean, approximately 40 % (Sigma; P-3644); PC from dried egg yolk, approximately 60 % (Sigma; P-5394); synthetic dioleylphosphatidylcholine (DOPC), approximately 99 % (Sigma; P-6354); PC from soybean, approximately 70 % (Lipoid S75); and PC from soybean, approximately 94 % (Lipoid S100). PBS is phosphate-buffered saline from Invitrogen Corp. (Dulbecco's; cat. no. 14190-094).

All the investigated polar lipids have chain melting phase transition temperatures well below 0°C, i.e., in the range of –10 to -15°C, when fully hydrated.

The peptide LL-37 and the lipid carrier were weighed in a 100 ml glass flask and then PBS was added. The total volume was about 30 ml. The samples were 5 vigorously shaken, using an ST mixer (type B1, E. Büchler, Tübingen) set at 5.5 (corresponding to an approximate frequency of 150/min) for 2 h, and allowed to equilibrate and settle for about 30 min at room temperature. The turbidity of the resulting dispersions was then recorded at 400-800 nm on a Shimadzu UV-VIS Spectrophotometer UV-160A. The measurements were made against pure water at room temperature 10 using a 10 mm cuvette cell. Turbidity data in Table 3 are presented as % transmission at 600 nm. Visual assessments of the dispersions were also made. Turbidity measurements were repeated after one and two days of storage at room temperature of the dispersions.

Table 3. Turbidity data

| Turbidity<br>( $\lambda = 600$ nm) | Sample D | Sample E | Sample F | Sample G | Sample H | Sample I | Sample J |
|------------------------------------|----------|----------|----------|----------|----------|----------|----------|
| 30 min                             | 64.1 %   | 70.9 %   | 5.1 %    | 1.7 %    | 68.6 %   | 18.6 %   | 1.4 %    |
| 1 day                              | 57.3 %   | 65.6 %   | -        | -        | 67.0 %   | 19.8 %   | -        |
| 2 days                             | 57.2 %   | 65.5 %   | -        | -        | 66.9 %   | 20.5 %   | -        |

15

From the visual assessments it was concluded that all mixtures formed more or less turbid dispersions; samples D, E, H, and I formed the least turbid dispersions, manifested in the highest transmission of light in Table 3, whereas samples F, G, and J formed the most turbid dispersions and consequently gave rise to the lowest transmission of light detected by the spectrophotometer. After one day of storage at room temperature, samples F, G, and J with the initially high turbidity (low transmission) had all sedimented and were not measured. Samples D, E, H, and I were all stable dispersions and resulted in reproducible turbidity data, after one and two days after preparation.

25

Samples D and H are duplicates, both containing CPL-Galactolipid but sample H had a slightly higher weight ratio of peptide to galactolipid. This resulted in a slightly lower turbidity (higher transmission) in sample H suggesting that the interaction between peptide and lipid in this sample is stronger than that in sample D, leading to smaller complexes/aggregates which give rise to lower turbidity.

Samples D, E, H, and I were further monitored with respect to colloidal stability at 2-8°C for 2 months.

Table 4. Stability data

| Sample | Appearance  | Stability      |
|--------|---|----------------|
| D      | fine turbid dispersion, slight sedimentation, easy to redisperse sediment | acceptable     |
| E      | turbid dispersion, slight sedimentation; microbial growth                 | not acceptable |
| H      | fine turbid dispersion, slight sedimentation, easy to redisperse sediment | acceptable     |
| I      | turbid dispersion, slight sedimentation; microbial growth                 | not acceptable |

5 These data and observations show that two mixtures of peptide and polar lipid carrier are better than the rest of the tested mixtures. The carriers containing CPL-Galactolipid (sample D and H) and PC from soybean, ca 40 % (sample E) gave rise to the most finely dispersed systems with the longest colloidal stability; however, it is only CPL-Galactolipid which is acceptable for pharmaceutical use, since the phospholipid 10 material with only 40 % phosphatidylcholine may be used for technical applications only. These data again demonstrate the usefulness of the galactolipid material in various pharmaceutical applications, e.g. as a carrier system for peptides.

15 Example 5. Preparation of aqueous mixtures comprising varying contents of LL-37 peptide and varying contents of galactolipid

A stock solution of LL-37 peptide (acetate salt; lot 990/37/A) in PBS, 995 ppm, and a stock solution of CPL-Galactolipid, 1.00 %, in PBS were prepared. Aliquots of the stock solutions plus additional PBS were mixed in 20 ml glass vials with rubber stoppers and aluminum caps. The compositions of the mixtures are presented in Table 20 5. After equilibration at room temperature for 1 h, the vials were shaken in horizontal position on an ST mixer (type B1, E. Büchler, Tübingen), set at 7.5 (corresponding to an approximate frequency of 190/min), for 1 h. The mixtures were then allowed to equilibrate and settle over night at room temperature. The appearances of the mixtures after one and five days at 4°C were evaluated as: clear colloidal, slightly turbid, turbid, 25 milky, and the results are summarized in Table 5.

Table 5.

| Sample number | LL-37 (ppm) | Galacto-lipid (%) | Peptide: Lipid (w/w) | Appearance after 1 day                  | Appearance after 5 days                   |
|---------------|-------------|-------------------|----------------------|---|---|
| 01            | 247         | 0.135             | 1:5.5                | turbid dispersion, sediment             | turbid dispersion, sediment               |
| 02            | 181         | 0.133             | 1:7.4                | clear colloidal solution                | clear colloidal solution, slight sediment |
| 03            | 116         | 0.133             | 1:11                 | clear colloidal solution                | clear colloidal solution                  |
| 04            | 50.5        | 0.135             | 1:27                 | clear colloidal solution                | clear colloidal solution                  |
| 05            | 16.5        | 0.133             | 1:81                 | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 06            | 8.2         | 0.135             | 1:165                | turbid dispersion, homogeneous          | turbid dispersion, homogeneous            |
| 07            | -           | 0.133             | -                    | turbid dispersion, homogeneous          | turbid dispersion, homogeneous            |
| 08            | 248         | 0.266             | 1:11                 | clear colloidal solution                | clear colloidal solution, slight sediment |
| 09            | 182         | 0.267             | 1:15                 | clear colloidal solution                | clear colloidal solution                  |
| 10            | 116         | 0.266             | 1:23                 | clear colloidal solution                | clear colloidal solution                  |
| 11            | 49.8        | 0.268             | 1:54                 | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 12            | 17.1        | 0.266             | 1:156                | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 13            | 8.9         | 0.265             | 1:298                | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 14            | -           | 0.265             | -                    | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 15            | 247         | 0.532             | 1:22                 | clear colloidal solution                | clear colloidal solution                  |
| 16            | 182         | 0.532             | 1:29                 | slightly turbid dispersion, homogeneous | slightly turbid dispersion, homogeneous   |
| 17            | 116         | 0.533             | 1:46                 | turbid dispersion, homogeneous          | turbid dispersion, homogeneous            |
| 18            | 49.2        | 0.533             | 1:108                | turbid dispersion, homogeneous          | turbid dispersion, homogeneous            |

|    |      |       |       |                                |                                    |
|----|------|-------|-------|--------------------------------|------------------------------------|
| 19 | 16.5 | 0.534 | 1:324 | turbid dispersion, homogeneous | turbid dispersion, homogeneous     |
| 20 | 8.2  | 0.532 | 1:649 | turbid dispersion, homogeneous | turbid dispersion, homogeneous     |
| 21 | -    | 0.533 | -     | turbid dispersion, homogeneous | turbid dispersion, homogeneous     |
| 22 | 248  | 0.799 | 1:32  | turbid dispersion, homogeneous | turbid dispersion, slight sediment |
| 23 | 182  | 0.802 | 1:44  | milky dispersion, homogeneous  | milky dispersion, slight sediment  |
| 24 | 115  | 0.801 | 1:70  | milky dispersion, homogeneous  | milky dispersion, slight sediment  |
| 25 | 50.1 | 0.799 | 1:159 | milky dispersion, homogeneous  | milky dispersion, slight sediment  |
| 26 | 16.8 | 0.799 | 1:476 | milky dispersion, homogeneous  | milky dispersion, slight sediment  |
| 27 | 8.6  | 0.798 | 1:928 | milky dispersion, homogeneous  | milky dispersion, slight sediment  |
| 28 | -    | 0.798 | -     | milky dispersion, homogeneous  | milky dispersion, slight sediment  |

It is clear that certain ratios of LL-37 peptide and galactolipid give rise to an appearance in solution, which indicate the presence of small complexes, smaller in size than particles of the corresponding samples without LL-37. A clear solution indicates a superior colloidal stability.

#### Example 6. Conformational measurements

Measurements of circular dichroism (CD) of LL-37 in solution may reveal information about conformational changes. The antibacterial activity of LL-37 is dependent on the conformation: a high content of helical content results in a strong antibacterial action and a high cytotoxic activity (Johansson *et al.*, *J Biol Chem* 273:3718-3724, 1998). It has been found that the  $\alpha$ -helical conformation of LL-37 is dependent on the counterion, the pH, and the peptide concentration (Johansson *et al.*, *J Biol Chem* 273:3718-3724, 1998). It is also known that a certain fraction of the peptide

has an  $\alpha$ -helical structure in aqueous solution and that this structure may be promoted by the presence of additives such as lipids, transforming it from a random coil to an  $\alpha$ -helix (Turner *et al.*, *Antimicrob Agents Chemother* 42:2206-2214, 1998).

Samples for circular dichroism (CD) measurements were prepared in 10 mM aqueous phosphate buffer solution, pH 7.0, containing 200 ppm LL-37 (as the trifluoroacetate, lot 971/26), with and without 0.40 % CPL-Galactolipid. The samples, 20 ml in 50 ml glass flasks, were vigorously shaken with an ST mixer (type B1, E. Büchler, Tübingen) set at 7.5 (corresponding to an approximate frequency of 220/min) for 2 h. They were then allowed to equilibrate and settle over night at 2-8°C.

CD spectra were recorded on a Jasco J-720 (Jasco Inc.) spectropolarimeter. The sample compartment with the cuvette cell (1 mm path length) was placed near the photomultiplier, in order to reduce effects of light scattering from the dispersions. The samples were measured at room temperature and scanned from 280 to 200 nm at a rate of 20 nm/min, with a resolution of 1 nm and 3 accumulations per run. The results are expressed as the mean residue ellipticity,  $[\theta]$ , and the percentage of  $\alpha$ -helical conformation at 222 nm is estimated by the following formula:  $([\theta]_{222} + 3900) \cdot 100 / 41900$ .

The CD measurements on 200 ppm LL-37 in 10 mM phosphate buffer solution, pH 7.0, revealed an  $\alpha$ -helical secondary structure by double dichroic minima at 208 and 222 nm. The minimum at 222 nm was used to calculate the percentage  $\alpha$ -helical structure, which was found to be about 63 %. When the galactolipid was added at a concentration of 0.40 % (w/w) in the same buffer solution the  $\alpha$ -helical structure of LL-37 was practically unaffected, with an approximate  $\alpha$ -helical structure of 64 %.

Enhanced helical conformation is related to increased antibacterial activity. It is speculated that the secondary structure is also relevant for the wound healing capacity of LL-37, where a high percentage of  $\alpha$ -helical structure means enhanced activity. In an aqueous buffer solution this also means high cytotoxicity, but in the presence of galactolipid the secondary structure is retained, and thus the activity is unaffected, whereas the cytotoxicity is diminished.

An anionic synthetic phospholipid, palmitoyl-oleoyl-phosphatidylglycerol (POPG; Sigma-Aldrich, P6956) was used as a reference and tested using the same experimental conditions as described above. A lower percentage of  $\alpha$ -helical structure, 58 %, was found when this lipid was present, indicating that the conformation and thus

activity of LL-37 is more influenced by the negatively charged phospholipid than by the neutral galactolipid. However, more importantly, after one month of storage at 4°C the sample had partially separated, with sediments on the bottom of the container. Gentle shaking resulted in a coarse dispersion. At the same time-point, sediments were also observed in the corresponding sample based on galactolipid, but to a lesser extent, which could be redispersed to a fine dispersion by gentle agitation.

#### Example 7. Cytotoxicity tests

10 *In vitro* cytotoxicity assays are valuable for the evaluation of the toxicity of materials, which come into close contact with living tissues.

Selected formulations were tested for *in vitro* cytotoxicity in cultured mammalian cells (L 929 mouse fibroblasts). The test design was based on the US Pharmacopeia 26<sup>th</sup> edition, Method <87> and the ISO 10993-5 standard.

15 Formulations D and E (see Example 4, Table 2) were mixed with complete cell culture medium (HAM F12 medium with 10 % foetal bovine serum) at concentrations of 10, 2, 0.4 and 0.08 % (v/v). These test solutions were used to treat triplicate cell cultures for 24 h. Triplicate untreated cultures, negative controls (treated with an extract of polypropylene) and positive controls (treated with an extract of tin-stabilised polyvinyl chloride) were included.

20 Both formulations showed no to slight toxicity (cytotoxicity grade 0-1) when tested at 10 % (v/v) and no toxicity (cytotoxicity grade 0) at 2 %, 0.4 % and 0.08 % (v/v).

25 Cytotoxicity test with a positive control solution containing 100 ppm LL-37 in PBS caused mild toxicity (cytotoxicity grade 2) at all four concentrations tested (10, 2, 0.4 and 0.08 % mixtures of the solution with cell culture medium). This level of toxicity is defined as 20-50 % of the cells being dead or showing morphological signs of toxicity. The scale has a range of 0 to 4 and when test extracts of medical devices are tested, grades 3 and 4 fail the test. This positive control solution is considerably more toxic than formulation D and E which showed no or just slight toxicity.

#### 30 BIOLOGICAL EXPERIMENTS

Based on our recent findings that

- hCAP18/LL-37 is induced in skin and mucous membranes in association with inflammation and wounding, and
- hCAP18/LL-37 is lacking in chronic ulcer epithelium despite massive inflammation,

we hypothesized that hCAP18/LL-37 is involved in the regenerative capacity of skin epithelium. The following experiments were performed to test this hypothesis.

Test 1. Investigation of the expression pattern of hCAP18/LL-37 in non-inflammatory

5 human wound healing

*Tissue samples*

Human skin was obtained from routine abdominal or breast reduction surgery. Under sterile conditions, full-thickness wounds were made, on the epidermal side, with a 3-mm biopsy punch. These *ex vivo* wounds were excised with a 6-mm biopsy punch and subsequently transferred to 24-well plates and covered with 2 ml of medium. Such wounds reproducibly re-epithelialize within 4-7 days (Kratz *et al.* *Scand J Plast Reconstr Surg Hand Surg* 28:107-112, 1994; Inoue *et al.*, *J Invest Dermatol* 104:479-483, 1995; Kratz *et al.*, *Microsc Res Tech* 42:345-350, 1998). Medium, DMEM (Dulbecco's modified Eagle's medium, GIBCO) containing 10 % fetal calf serum (FCS) and antibiotics (PEST = penicillin 50 U/ml and streptomycin 50 mg/ml), was changed every third day. Wounds were harvested at different time-points, by 2, 4 and 7 days post-wounding and snap frozen. In total, the experiment was repeated four times. Four different donors were used and triplicate wounds were made for each condition in every experiment. In each experiment, only skin from a single donor was used.

20 *Preparation of RNA probes*

To detect mRNA for the hCAP18 gene and immunoreactivity for hCAP18/LL-37 we performed *in situ* hybridization and immunohisto-chemistry on samples of wounds representing all time-points of sequential re-epithelialization. For *in situ* hybridization we used <sup>35</sup>S-labeled antisense and sense RNA probes and the experiment was performed as described (Frohm Nilsson *et al.*, *Infect Immun* 67:2561-2566, 1999).

*Preparation of LL-37 antibody*

For immunohistochemistry we raised and prepared a polyclonal LL-37 antibody as follows: LL-37 peptide (lot YS 5253, EuroDiagnostica AB, Malmö, Sweden) was prepared as a trifluoroacetate salt according to Fmoc-strategy using solid phase synthesis (Fields and Noble, 1990) and purified by HPLC to a purity of 98 %. Biological activity of the peptide was confirmed in an antibacterial assay. The peptide was used for immunization of three rabbits according to a standard protocol (AgriSera, Vännäs, Sweden). Polyclonal antiserum was affinity-purified using synthetic LL-37 peptide and

the purified antiserum was assessed with ELISA. IgG concentration of the immune serum was diluted to 0.5 mg/ml. Pre-immune serum was collected from each rabbit and the IgG concentration was 2 mg/ml.

#### *Immunohistochemistry*

5 All biopsies were snap frozen and handled identically. In short, 6-7 µm thick cryostat sections were incubated with the LL-37 antibody at dilutions 1:1000 and 1:2000 and stained according to the indirect peroxidase method using a Vectastain kit (Vector Laboratories, Burlingame, USA) and following the manufacturer's instructions. Sections were counterstained with Mayer's hematoxyline solution. All experiments were repeated  
10 minimum three times to ensure reproducability. As controls, serial tissue sections were processed in parallel without adding primary antibody and using pre-immune rabbit IgG (DAKO, Glostrup, Denmark) as primary antibody.

#### *Results*

At time-point 0 h there was moderate expression of hCAP18 mRNA and LL-  
15 37 protein in the basal layer of the epidermis throughout the tissue consistent with our previous findings of a constitutive hCAP18 expression in basal epidermis. Wounds harvested at different time-points during re-epithelialization demonstrated a distinct signal for hCAP18 mRNA and LL-37 protein in the epithelium migrating to cover the wounded surface. No cells in the underlying dermal matrix were positive for hCAP18/LL-  
20 37. These results indicate that de novo synthesis of hCAP18 occurs in keratinocytes during re-epithelialization without inflammation and support our hypothesis that hCAP18 may be linked to epithelial regeneration.

#### Test 2. Inhibition of re-epithelializing of human skin wounds *ex vivo* with LL-37 antibody.

25 LL-37 antibody, prepared in Test 1, was added in 2 ml medium per well (DMEM, + 10 % FCS and PEST) to a final antibody dilution of 1:10, 1:100 and 1:1000. As control we used the corresponding pre-immune serum at a final IgG concentration equal to the 1:10 dilution of the LL-37 antiserum and a set of wounds treated only with medium. Each experimental condition was made in triplicates and repeated twice. The  
30 media were changed every third day and LL-37 antibody or pre-immune serum was added as described above. The *ex vivo* wounds were harvested 2, 4 and 7 days post-wounding. All specimens were snap frozen, sectioned in completion and mounted on Superfrost Plus slides prior to staining with hematoxylin-eosine. Sections representing maximal re-epithelialization in the center of the wounds were selected for evaluation.

The proliferative capacity of keratinocytes was investigated through immunohistochemistry with the proliferation marker Ki67 (mouse monoclonal Ki67 antiserum (DAKO, Glostrup, Denmark) at 1:25 dilution) in wounds representing all treatment conditions.

### Results

5 Treatment with LL-37 antibody produced a concentration-dependant inhibition of re-epithelialization. All wounds treated with the highest LL-37 antibody concentration (1:10) failed to re-epithelialize. In these wounds only single keratinocytes with a fragile flattened appearance had migrated from each wound edge. The wounds treated with LL-37 at medium concentration (1:100) showed delayed re-epithelialization, 10 these wounds were mostly healed by day 7 but not by day 4. Moreover, the epithelium was thinner and the keratinocytes had a fragile appearance. Wounds treated with LL-37 antibody at the lowest concentration (1:1000) did not differ from control wounds, which had all healed by day 4 with a 2-3 layer robust epithelium. Control wounds treated with only medium and control IgG antibody healed equally. In the control wounds the 15 majority of cells in the re-epithelializing tongue were positive for the proliferation marker Ki67, whereas there were no Ki67 positive cells in the wounds treated with LL-37 at 1:10. We concluded from this experiment that LL-37 may be critically involved in skin re-epithelialization and that the proliferative capacity seemed preferentially affected, since blocking with LL-37 antibody allowed the initial migration of single cells from the wound 20 edge, but effectively prevented further proliferation of the keratinocytes.

### Test 3. Proliferation of HaCat cells by treatment with synthetic, biologically active LL-37 peptide per se and in combination with a polar lipid carrier.

HaCat cells were used for these experiments. HaCat cells are an 25 immortalized human keratinocyte cell line (Boukamp *et al.*, *J Cell Biol* 106:761-771, 1988), which is suitable for experimental keratinocyte research. HaCat cells were cultured in medium (DMEM, + 10 % FCS and PEST). Both types of cell cultures were treated with synthetic, bioactive LL-37 (lot YS 5253). In addition a mixture of LL-37 (114 µg/ml) and CPL-Galactolipid (0.2 %) in medium containing serum at either 2 or 10 % 30 was added to evaluate the capacity to increase proliferation and inhibit cytotoxicity. Cells were harvested at different time-points (24 h, 48 h, 72 h and 96 h) and counted by flowcytometry (Becton-Dickinson) and stained by Trypan-Blue to evaluate viability. Positivity for Trypan-Blue indicates that the cell membrane has been damaged.

Proliferation and viability were also ascertained by measuring mitochondrial activity (WST-1, Roche, Cook *et al. Anal Biochem* 179:1-7, 1989).

Table 6. Proliferation of HaCat cells at 96 h assessed by flowcytometry.

| EGF (nM) | LL-37 (µg/ml) | Serum Conc. (%) | Number of Cells (Mean) | Trypan Blue + (%) | Increased Proliferation (%)      |
|----------|---------------|-----------------|------------------------|-------------------|----------------------------------|
| -        | -             | 10              | 32270                  | < 1               | 0                                |
| 1.7      | -             | 10              | 42000                  | < 1               | 30                               |
| -        | 25            | 10              | 36470                  | < 1               | 13                               |
| -        | 50            | 10              | 40950                  | < 1               | 27                               |
| -        | 100           | 10              | 66430                  | < 1               | 100                              |
| -        | 25            | 2               | 32130                  | < 1               | 0                                |
| -        | 50            | 2               | 53620                  | 30-50             | Not relevant<br>Cytotoxic effect |
| -        | 100           | 2               | 15120                  | 100               | Not relevant<br>Cytotoxic effect |

5 Increase in cell proliferation is calculated in comparison with baseline (-EGF). Mean values from triplicate samples/condition in three separate experiments are presented

10 Table 7. Proliferation and viability of HaCat cells at 48 h measured by mitochondrial activity (WST-1).

| EGF (nM) | LL-37 (µg/ml) | Serum Conc. (%) | Absorbance | Trypan Blue + (%) | Increased Proliferation (%) |
|----------|---------------|-----------------|------------|-------------------|-----------------------------|
| -        | -             | 10              | 0.622      | < 1               | 0                           |
| 1.7      | -             | 10              | 1.107      | < 1               | 77                          |
| -        | 100           | 10              | 1.110      | < 1               | 78                          |

15 Increase in cell proliferation is calculated in comparison with baseline (-EGF). Mean values from 6 samples/condition in one experiment are presented

Table 8. Proliferation of HaCat cells at 72 h assessed by flowcytometry.

| EGF (nM) | LL-37 ( $\mu$ g/ml) | Lipid (0.2%) | Serum Conc. (%) | Number of Cells (Mean) | Trypan Blue + (%) | Increased Proliferation (%)   |
|----------|---------------------|--------------|-----------------|------------------------|-------------------|-------------------------------|
| -        | -                   | -            | 10              | 55207                  | < 1               | 0                             |
| 1.7      | -                   | -            | 10              | 85050                  | < 1               | 54                            |
| 1.7      | -                   | +            | 10              | 87640                  | < 1               | 58                            |
| -        | 100                 | -            | 10              | 88853                  | < 1               | 61                            |
| -        | 100                 | +            | 10              | 91980                  | < 1               | 66                            |
| -        | 100                 | -            | 2               | 150500                 | 100               | Not relevant Cytotoxic effect |
| -        | 100                 | +            | 2               | 87360                  | < 1               | 58                            |

Increase in proliferation is calculated in comparison with baseline (-EGF). Mean values from triplicate samples / condition in one experiment are presented.

## 5 Results

The treatment of HaCat cells with LL-37 peptide resulted in a concentration-dependant increase in proliferation. This indicates that LL-37 peptide has the capacity to stimulate the proliferation of keratinocytes to a level that equals or surpasses that of EGF, the golden standard for epithelial cell proliferation. We have used EGF at 1.7 nM since this has been established as optimal to stimulate proliferation of keratinocytes in culture and has become a standard culture condition (Cohen *et al.*, *Dev Biol* 12:394-407, 1965). HaCat cells are highly proliferating epithelial cells and it is interesting that LL-37 can increase the proliferation of these cells even further. The cytotoxic effect induced by LL-37 at 100  $\mu$ g/ml, in 2 % serum was completely abolished when lipid was added to the mixture, indicating that the lipid is able to substitute for serum in this experimental condition.

The test has shown that synthetic, bioactive LL-37 (25 -100  $\mu$ g/ml) added to cell cultures of HaCat cells, in media with 10 % Fetal Calf Serum (FCS), increases proliferation in a concentration-dependent manner. However, if the peptide (100  $\mu$ g/ml) was added to a keratinocyte culture in a medium containing 2 % FCS, all of the keratinocytes became positive with Trypan Blue staining, indicating a cytotoxic effect on these cells.

The cytotoxic activity of cathelicidin is inhibited by the presence of serum, a mechanism thought to protect the host cells from potentially harmful effects. Our data confirms that the cytotoxic effect of LL-37 is inhibited in the presence of serum (10 %). In addition, the mixture of LL-37 (25  $\mu$ M) and polar lipid carrier (0.2 %), in medium containing the lower serum concentration (2 % FCS), inhibits the cytotoxic effect and

increases the proliferation. These data suggest that the polar lipid carrier has similar protecting capacity as serum, without interfering with the LL-37 bioactivity.

Primary data show that human keratinocytes are proliferated in the same way as HaCat cells.

5

Test 4. Proliferation of HaCat cells by treatment with the synthetic peptides LL-36, LL-37 and LL-38

HaCat cells were cultured in medium (DMEM, + 10 % FCS and PEST). HaCaT cells were plated in 96 well plates (Falcon, USA) at the concentration of 2000 cells per well. Cells were plated at -48 hours and stimulated with different concentrations of synthetic LL-37, LL-36, and LL-38 peptide by hour 0 and after 48 hours.

The testing was done in one experiment with 6 wells in each condition. 1 Ci/mmol of  $^3$ H-Thymidine (THYMIDINE, [METHYL- $^3$ H]-740.0 GBq/mmol (20.00 Ci/mmol) 1.0 ml of Ethanol:Water, 7:3, Perkin Elmer Life Sciences Inc. Boston MA., USA) was added to each well and incubated for 12-17 hours. Proliferation was evaluated by  $^3$ H-Thymidine incorporation a liquid scintilator (MicroBeta Perkin Elmer Life Sciences Inc. Boston MA., USA) after 72 and 96 hours.

20 Table 9. Proliferation of HaCat cells by LL-37 at 96 h assessed by  $^3$ H-Thymidine incorporation after 72 and 96 hours.

| LL-37<br>( $\mu$ g/ml) | Serum<br>Conc.<br>(%) | Counts Per<br>Minute<br>(Mean) | Standard<br>Deviation<br>(+/-) | Increased<br>Proliferation<br>(%) |
|------------------------|-----------------------|--------------------------------|--------------------------------|-----------------------------------|
| 0                      | 10                    | 52774                          | 11639                          | 0                                 |
| 1.00                   | 10                    | 75445                          | 32827                          | 43                                |
| 5.00                   | 10                    | 102353                         | 33808                          | 94                                |
| 10.00                  | 10                    | 73548                          | 8424                           | 39                                |
| 25.00                  | 10                    | 76510                          | 10550                          | 45                                |
| 50.00                  | 10                    | 65119                          | 8565                           | 23                                |

25 Increase in cell proliferation (Proliferation Index) is calculated in comparison with baseline (Control = 0  $\mu$ g/ml). Mean values from four samples per condition in one experiment are presented.

Table 10. HaCat cells stimulated by LL-36 peptide. Proliferation assessed by  $^3\text{H}$ -Thymidine incorporation after 96 hours.

| LL-36<br>( $\mu\text{g/ml}$ ) | Serum<br>Conc.<br>(%) | Counts Per<br>Minute<br>(Mean) | Standard<br>Deviation<br>(+/-) | Increased<br>Proliferation<br>(%) |
|-------------------------------|-----------------------|--------------------------------|--------------------------------|-----------------------------------|
| 0                             | 10                    | 69323                          | 7511                           | 0                                 |
| 1.00                          | 10                    | 86253                          | 10770                          | 24                                |
| 5.00                          | 10                    | 116381                         | 14570                          | 68                                |
| 10.00                         | 10                    | 70157                          | 3660                           | 1                                 |
| 25.00                         | 10                    | 72674                          | 7965                           | 5                                 |
| 50.00                         | 10                    | 68560                          | 11699                          | -1                                |

Increase in cell proliferation (Proliferation Index) is calculated in comparison with baseline (Control = 0  $\mu\text{g/ml}$ ). Mean values from four samples per condition in one experiment are presented.

Table 11. HaCat cells stimulated by LL-38 peptide. Proliferation assessed by  $^3\text{H}$ -Thymidine incorporation after 96 hours.

| LL-38<br>( $\mu\text{g/ml}$ ) | Serum<br>Conc.<br>(%) | Counts Per<br>Minute<br>(Mean) | Standard<br>Deviation<br>(+/-) | Increased<br>Proliferation<br>(%) |
|-------------------------------|-----------------------|--------------------------------|--------------------------------|-----------------------------------|
| 0                             | 10                    | 79191                          | 15277                          | 0                                 |
| 1.00                          | 10                    | 82008                          | 7911                           | 4                                 |
| 5.00                          | 10                    | 68694                          | 16599                          | -13                               |
| 10.00                         | 10                    | 57293                          | 8512                           | -28                               |
| 25.00                         | 10                    | 54294                          | 14335                          | -31                               |
| 50.00                         | 10                    | 48701                          | 6080                           | -39                               |

Increase in cell proliferation (Proliferation Index) is calculated in comparison with baseline (Control = 0  $\mu\text{g/ml}$ ). Mean values from four samples per condition in one experiment are presented.

#### Test 5. Proliferation of human fibroblasts by treatment with LL-37 peptide

The peptide LL-37 used in this and following tests was as described in

Example 1 (lot 971/26). The fibroblasts, a type of stromal cells, were obtained from injured and uninjured skin in patients with chronic leg ulcers due to venous insufficiency. Punch-biopsies (4-mm) were taken from the wound margin including 50 % of the epithelialized area and from uninjured skin in the knee region. Individuals with a history of diabetes mellitus, arterial insufficiency or chronic inflammatory disease were excluded. Patients with signs of eczema in the ulcer margin, clinical signs of infection or undergoing systemic or topical antibiotic treatment at the time for biopsy were also

excluded. Patients included were all treated with inert local dressings and standard compression bandaging.

Fibroblast were put in culture using explant technique (Hehenberger *et al.*, *Cell Biochem Funct* 15:197-201, 1997). Fibroblasts were plated in 96 well plates (Falcon, USA) at the concentration of 2000 cells per well. Cells were plated at -48 hours and stimulated with different concentrations of synthetic LL-37 peptide by hour 0. The testing was done in one experiment with 6 wells in each condition. Proliferation and viability were ascertained by measuring mitochondrial activity (WST-1, Roche) after 24 h and 48 h. See Table 12 and Table 13 below. Increase in cell proliferation (Proliferation Index) is calculated in comparison with baseline (Control = 0 µg/ml). Mean values from six samples per condition in one experiment are presented.

Table 12. Human Wound Fibroblast stimulated by LL-37. Proliferation and viability of Human Fibroblasts measured by mitochondrial activity (WST-1) at 48 hours.

| LL-37<br>(µg/ml) | Lipid<br>(0.2%) | Serum<br>Conc.<br>(%) | Absorbance | Standard<br>Deviation<br>(+/-) | Increased<br>Proliferation<br>(%) |
|------------------|-----------------|-----------------------|------------|--------------------------------|-----------------------------------|
| -                | -               | 10                    | 0.785      | 0.020                          | 0                                 |
| 25               | -               | 10                    | 1.171      | 0.242                          | 49                                |
| 50               | -               | 10                    | 1.073      | 0.199                          | 37                                |
| 100              | -               | 10                    | 0.955      | 0.187                          | 22                                |
| 100              | +               | 2                     | 0.960      | 0.122                          | 22                                |

15

Table 13. Human Normal Fibroblast stimulated by LL-37 peptide. Proliferation and viability of Human Fibroblasts measured by mitochondrial activity (WST-1) at 48 hours.

| LL-37<br>(µg/ml) | Serum<br>Conc.<br>(%) | Absorbance | Standard<br>Deviation<br>(+/-) | Increased<br>Proliferation<br>(%) |
|------------------|-----------------------|------------|--------------------------------|-----------------------------------|
| -                | 10                    | 0.560      | 0.019                          | 0                                 |
| 25               | 10                    | 0.597      | 0.067                          | 7                                 |
| 50               | 10                    | 0.626      | 0.076                          | 12                                |
| 100              | 10                    | 0.669      | 0.051                          | 19                                |

20

Test 6. Transgenic expression of hCAP18 in HEK293 cells and proliferation of HEK293-hCAP18 cells

A *Bfa1* fragment from Image clone 3057931 (ref) containing the entire coding sequence for hCAP18 including the 16 bp of the 5'-untranslated region, was 5 subcloned into the *Sma1*-site of the bicistronic vector pIRES2-EGFP (BD Biosciences, Bedford, MA). Human embryonic kidney cells, HEK293, were transfected using Fugene (Roche Diagnostics, Indianapolis, IN) under standard conditions, and selected for two weeks with 400 ng/ml G418 antibioticum (Invitrogen, Paisley, UK). The cells were sorted for EGFP expression with a MoFlo® high speed cell sorting flow cytometer 10 (DakoCytomation, Fort Collins, CO) using Summit™ software for data analysis, and their expression of CAP18 was quantified by immunoblotting. Control cell lines were similarly established by transfection with the vector only expressing EGFP.

For proliferation assay, cell lines were harvested at 70% confluence and seeded in 24-well plates. After 24 hours, medium was changed and cells were cultured 15 in 2 ml of medium (OPTIMEM, Gibco BRL, Life Technologies, Scotland) supplemented with 5 % FCS and PEST. All conditions were performed in triplicates. Medium was changed every second day. Cell lines were then harvested at day 6 and counted by Flow Cytometry. Cell viability was measured with Trypan Blue; under all conditions < 5 % of the cells were Trypan Blue positive. Increase in cell proliferation (Proliferation 20 Index) is calculated in comparison with baseline (HEK293-EGFP). Mean values from triplicate samples per condition in one experiment are presented.

Table 14. Proliferation of HEK293-hCAP-18 cells at 144 hours assessed by flow-cytometry.

| Cell Type     | Serum Conc. (%) | Number of Cells (Mean) | Standard Deviation (+/-) | Increased Proliferation (%) |
|---------------|-----------------|------------------------|--------------------------|-----------------------------|
| HEK293-EGFP   | 5               | 169063                 | 63726                    | 0                           |
| HEK293-hCAP18 | 5               | 485884                 | 88168                    | 187                         |

25

The proliferation of the HEK293-hCAP18 cells was also assessed by incorporation of <sup>3</sup>H-thymidine and the results obtained are presented in Table 15 below. The increase in cell proliferation (Proliferation Index) is calculated in comparison with baseline (HEK293-EGFP). Mean values from four samples per condition in one 30 experiment are presented.

Table 15. Proliferation of HEK293-hCAP-18 cells at 144 hours assessed by  $^3\text{H}$ -Thymidine incorporation after 96 hours.

| Cell Type     | Serum Conc. (%) | Counts Per Minute (Mean) | Standard Deviation (+/-) | Increased Proliferation (%) |
|---------------|-----------------|--------------------------|--------------------------|-----------------------------|
| HEK293-EGFP   | 0.1             | 364                      | 118                      | 0                           |
| HEK293-hCAP18 | 0.1             | 796                      | 206                      | 111                         |
| HEK293-EGFP   | 0.5             | 811                      | 459                      | 0                           |
| HEK293-hCAP18 | 0.5             | 2271                     | 792                      | 180                         |
| HEK293-EGFP   | 1               | 744                      | 433                      | 0                           |
| HEK293-hCAP18 | 1               | 2303                     | 359                      | 209                         |
| HEK293-EGFP   | 2               | 767                      | 334                      | 0                           |
| HEK293-hCAP18 | 2               | 3483                     | 771                      | 354                         |
| HEK293-EGFP   | 5               | 958                      | 414                      | 0                           |
| HEK293-hCAP18 | 5               | 6088                     | 1783                     | 534                         |
| HEK293-EGFP   | 10              | 1806                     | 664                      | 0                           |
| HEK293-hCAP18 | 10              | 6541                     | 2827                     | 262                         |

5 Test 7. Culturing of human cells for transplantation in different growth media

*Culture of epithelial cells*

A piece of skin, 1X1 cm, is excised from healthy skin of the patient. The skin is minced and treated with trypsin/EDTA (0.05/0.01%) and  $2-5 \times 10^6$  of the recruited keratinocytes are added to  $1.5 \times 10^6$  mitomycin-pretreated (4  $\mu\text{g}/\text{ml}$ , 2h) 3T3 cells in  $75 \text{ cm}^2$  culture flasks. Growth medium A containing LL-37 peptide is added. Cells are harvested by trypsinization as sheets and transplanted onto the patient.

Growth Medium A is used for culture of epithelial cells such as e.g. keratinocytes *in vitro* and consists of Basal Medium and a growth promoting kit (GPK) including a) LL-37 peptide in a salt solution, b) penicillin + streptomycin, c) insulin, d) transferring, e) triiodothyronine, f) hydrocortisone, g) cholera toxin, and a selected cytotoxicity reducing agent, such as serum or a polar lipid.

*Culture of stromal cells*

Stromal cells are obtained from a 4 mm skin biopsy, cleaned from subcutaneous tissue and plated in cell culture dishes using the explant technique to obtain primary fibroblasts. Growth medium B is used for culturing the biopsy. Cells are harvested by trypsinization and given back to the patient.

Growth Medium B is used for culture of stromal cells such as e.g. fibroblasts *in vitro* and consists of Basal medium and a growth promoting kit including a) LL-37 peptide in a salt solution, b) penicillin + streptomycin, and a selected cytotoxicity reducing agent, such as serum or a polar lipid.

5 Basal medium is based on double-distilled water containing inorganic salts, phenol red, glucose, thymidine, hypoxanthine, HEPES, sodium pyruvate, aminopterin, amino acids and vitamins

#### SUMMARY OF THE EXPERIMENTS

10 In summary, it has been demonstrated that LL-37 is produced in skin epithelium during normal wound healing and that LL-37 is required for re-epithelialization to occur. We have also shown that endogenous LL-37 is lacking in chronic ulcer epithelium. We therefore propose that treatment with LL-37, as well as with N-terminal fragments of said peptide, and functional derivates thereof provides a rational strategy 15 to promote healing of such ulcers. Furthermore, addition of LL-37 and transgenic expression of hCAP18/LL-37 also stimulates proliferation of healthy cells indicating that LL-37 can be used to enhance bothg normal and deficient epithelial repair *in vivo* and proliferation of epithelial cells *in vitro* for autologous cell transplantation. We have also identified a suitable carrier and delivery system that reduces cytotoxicity and has the 20 potential to protect from rapid degradation *in vivo* of LL-37 and other cathelicidin peptides.

## CLAIMS

1. A peptide having a sequence of at least 20 amino acids of the N-terminal fragment of LL-37, SEQ ID NO 1, with the proviso that LL-37 is excluded, as well as pharmaceutically acceptable salts and derivatives thereof.  
5
2. A peptide according to claim 1, wherein 1-3 amino acids have been added to the C-terminal end of the sequence of LL-37, SEQ ID NO 1.
- 10 3. A peptide according to claim 1, selected from the group consisting of LL-36, LL-35, LL-34, LL-33, LL-32, LL-31, LL-30, LL-29, LL-28, LL-27, LL-26, LL-25, LL-24, LL-23, LL-22, LL-21 and LL-20, having the sequence SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, 15 SEQ ID NO 16, SEQ ID NO 17, and SEQ ID NO 18, respectively.
4. The peptide according to any of claims 1 - 3 for use as a medicament.
- 20 5. The peptide according to any of claims 1 - 3 for the preparation of a medicament for healing of wounds, cell proliferation, epithelial regeneration, and for use as antimicrobial agent.
6. Use of a peptide having an amino acid sequence selected from the group consisting of  
25 a) SEQ ID NO 1;  
b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;  
and pharmaceutically acceptable salts and derivatives thereof, which peptide enhances proliferation of epithelial and/or stromal cells through a non-lytic mechanism, for the preparation of a medicament for epithelial regeneration, and healing of wound epithelium and stroma.  
30
7. Use according to claim 6 of a peptide, which is LL-37, SEQ ID NO 1, in the form of an acetate salt.

8. Use according to claim 6 of a peptide selected from the group consisting of LL-36, LL-35, LL-34, LL-33, LL-32, LL-31, LL-30, LL-29, LL-28, LL-27, LL-26, LL-25, LL-24, LL-23, LL-22, LL-21 and LL-20, having the sequence SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, and SEQ ID NO 18, respectively.

5 9. Use according to any of claims 6 – 8 of a peptide, for the preparation of a medicament for the treatment of chronic ulcers due to venous insufficiency, metabolic 10 dysfunction, or immunological dysregulation.

10 10. Use according to any of claims 6 – 8 of a peptide, for the preparation of a medicament for the treatment of wounds due to trauma or burns.

15 11. A pharmaceutical composition comprising an anti-microbial cathelicidin peptide in the form of pharmaceutically acceptable salts or derivatives thereof in combination with a carrier consisting of a bilayer-forming polar lipid and an aqueous solution.

20 12. A pharmaceutical composition comprising a peptide having an amino acid sequence selected from the group consisting of

- a) SEQ ID NO 1;
- b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;

25 in the form of pharmaceutically acceptable salts or derivatives thereof in combination with a carrier consisting of a bilayer-forming polar lipid and an aqueous solution.

13. The pharmaceutical composition according to claim 11 or 12, wherein the bilayer-forming polar lipid is selected from the group consisting of phospholipids, galactolipids and sphingolipids.

30

14. The pharmaceutical composition according to any of claims 11 - 13, wherein the peptide is in the form of acetate.

15. The pharmaceutical composition according to any of claims 11 - 14, wherein the bilayer-forming polar lipid contains at least 50 % (w/w) of digalactosyldiacylglycerols.
16. The pharmaceutical composition according to any of the claims 12 - 15, wherein the 5 peptide is LL-37 in the form of an acetate salt.
17. The pharmaceutical composition according to any of claims 11 - 16, comprising a complex of an acetate of LL-37 in combination with CPL-Galactolipid.
18. The pharmaceutical composition according to any of claims 11 - 17, wherein the 10 ratio between the peptide as a salt and a galactolipid carrier is 1:10 – 1:50 by weight.
19. Use of a peptide having an amino acid sequence selected from the group consisting of 15
  - a) SEQ ID NO 1;
  - b) a sequence containing at least 20 amino acids of the N-terminal fragment of SEQ ID NO 1;in the form of pharmaceutically acceptable salts or derivatives thereof for proliferation of epithelial and/or stromal cells *in vitro* through a non-lytic mechanism.
20. Use according to claim 19 for proliferation of human autologous epithelial and 20 stromal cells *in vitro*.
21. A growth medium for culturing epithelial and/or stromal cells, comprising LL-37 or a 25 peptide as described in any of claims 1 - 3 in combination with a basal medium.
22. A growth medium for culturing epithelial and/or stromal cells, comprising LL-37 or a peptide as described in any of claims 1 - 3 in combination with a bilayer-forming polar 30 lipid in a basal medium.
23. The medium according to claim 22, wherein the polar lipid is selected from the group consisting of phospholipids, galactolipids and sphingolipids.
24. A method of enhancing the expansion of human autologous epithelial and stromal 35 cells *in vitro* for cell transplantation *in vivo*, wherein cells are isolated from an excised piece of healthy skin, said isolated cells are cultivated *in vitro* in a growth medium

according to any of claims 21 - 23, and the cultivated cells are subsequently harvested and used for treatment of wounds, such as burn injuries and ulcers.

25. A kit for culturing of epithelial and/or stromal cells comprising LL-37 or a peptide as described in any of claims 1 - 3 and a cytotoxicity reducing bilayer-forming polar lipid, optionally in combination with antibiotics, basal media, and other conventional additives.
26. Use of a gene construct comprising a cDNA sequence of hCAP18, SEQ ID NO 20, for transfection and transgenic expression of epithelial and/or stromal cells in order to enhance proliferation of said cells.

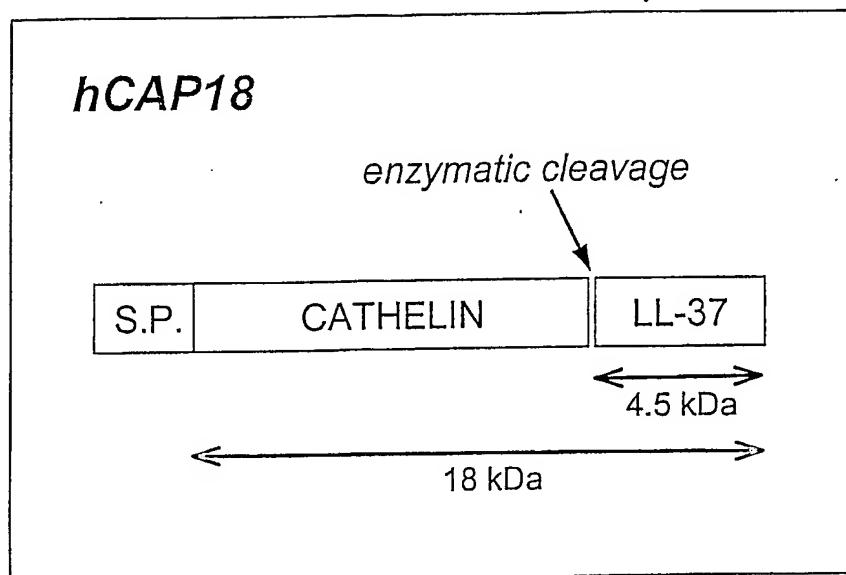


FIGURE 1

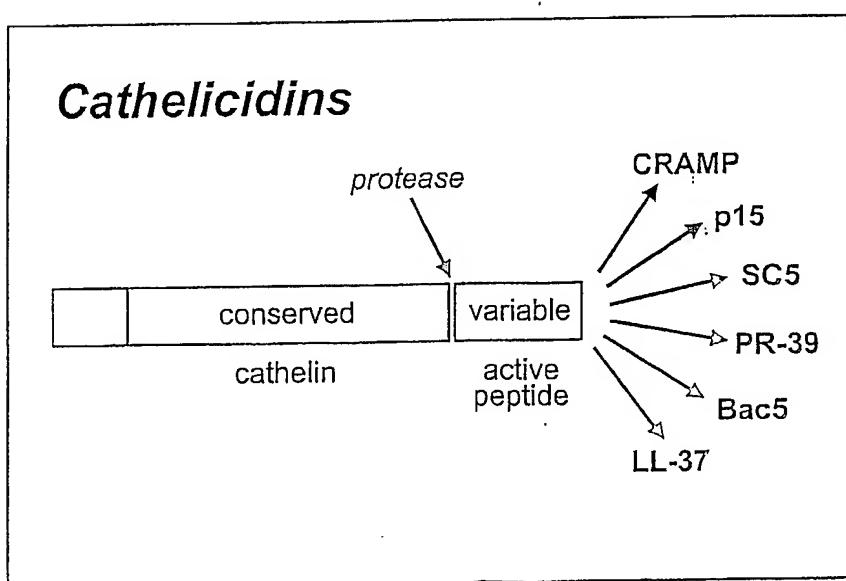


FIGURE 2

**The vector pIRES2-EGFP including the coding sequence for hCAP18**

TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTCAT AGCCCATAA  
 TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCGCC TGCTGACCG  
 CCCAACGACC CCCGCCATT GACGTCAATA ATGACGTATG TTCCCATAAGT  
 AACGCCATAA GGGACTTCC ATTGACGTCA ATGGGTGGAG TATTTACGGT  
 AAACTGCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCC  
 CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCAGTA  
 CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA  
 TCGCTATTAC CATGGTGTATG CGGTTTGGC AGTACATCAA TGGCGTGGA  
 TAGCGGTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA  
 TGGGAGTTG TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA  
 ACAACTCCGC CCCATTGACG CAAATGGCG GTAGGCGTGT ACGGTGGAG  
 GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGCGCTA  
 CCGGACTCAG ATCTCGAGCT CAAGCTTCGA ATTCTGCAGT CGACGGTACC  
 GCGGGCCCTA GAGGGAGGCA GACATGGGGA CCATGAAGAC CCAAAGGGAT  
 GGCCACTCCC TGGGGCGGTG GTCACTGGTG CTCCTGCTGC TGGGCCTGGT  
 GATGCCCTTG GCCATCATTG CCCAGGTCT CAGCTACAAG GAAGCTGTGC  
 TTCGTGCTAT AGATGGCATH AACCAGCGGT CCTCGGATGC TAACCTCTAC  
 CGCCTCCTGG ACCTGGACCC CAGGCCACG ATGGATGGNG ACCCAGACAC  
 GCCAAAGCCT GTGAGCTCA CAGTGAAGGA GACAGTGTGC CCCAGGACGA  
 CACAGCAGTC ACCAGAGGAT TGTGACTTCA AGAAGGACGG GCTGGTGAAG  
 CGGTGTATGG GGACAGTGAC CCTCAACCAG GCCAGGGCT CCTTGACAT  
 CAGTTGTGAT AAGGATAACA AGAGATTGC CCTGCTGGGT GATTTCTTCC  
 GGAAATCTAA AGAGAAGATT GGCAAAGAGT TTAAAAGAAT TGCCAGAGA  
 ATCAAGGATT TTTTGCGGAA TCTTGTACCC AGGACAGAGT CCTAGGGATC  
 CGCCCTCTC CCTCCCCCCC CCTTAACGTT ACTGGCCGAA GCCGCTTGGA  
 ATAAGGCCGG TGTGCGTTG TCTATATGTT ATTTTCCACC ATATTGCCGT  
 CTTTGGCAA TGTGAGGGCC CGGAAACCTG GCCCTGTCTT CTTGACGAGC  
 ATTCCCTAGGG GTCTTCCCC TCTCGCCAAA GGAATGCAAG GTCTGTTGAA  
 TGTGCGAAG GAAGCAGTTC CTCTGGAAGC TTCTGAAAGA CAAACAACGT  
 CTGTAGCGAC CCTTGCAGG CAGCGGAACC CCCCACCTGG CGACAGGTGC  
 CTCTGCGGCC AAAAGCCACG TGTATAAGAT ACACCTGCAA AGGCAGGCACA  
 ACCCCAGTGC CACGTTGTGA GTTGGATAGT TGTGGAAAGA GTCAAATGGC  
 TCTCCTCAAG CGTATTCAAC AAGGGGCTGA AGGATGCCA GAAGGTACCC  
 CATTGTATGG GATCTGATCT GGGGCCTCGG TGCACATGCT TTACATGTGT  
 TTAGTCGAGG TTAAAAAAAC GTCTAGGCC CCGAACAC GGGGACGTGG  
 TTTTCTTTG AAAAACACGA TGATAATATG GCCACAACCA TGGTGAGCAA  
 GGGCGAGGAG CTGTTCACCG GGGTGGTGCC CATCCTGGTC GAGCTGGACG  
 GCGACGTAAA CGGCCACAAG TTCAGCGTGT CGGGCGAGGG CGAGGGCGAT  
 GCCACCTACG GCAAGCTGAC CCTGAAGTTC ATCTGCACCA CCGGCAAGCT  
 GCCCGTGCC TGGCCCACCC TCGTGACCA CCTGACCTAC GCGTGCAGT  
 GCTTCAGCCG CTACCCGAC CACATGAAGC AGCACGACTT CTTCAAGTCC  
 GCCATGCCCG AAGGCTACGT CCAGGAGCGC ACCATCTTCT TCAAGGACGA  
 CGGCAACTAC AAGACCCGCG CCGAGGTGAA GTTCGAGGGC GACACCCCTGG  
 TGAACCGCAT CGAGCTGAAG GGCATCGACT TCAAGGAGGA CGGCAACATC

(cont. in Fig. 3B)

FIGURE 3A

(cont. from Fig. 3A)

CTGGGGCACA AGCTGGAGTA CAACTACAAC AGCCACAACG TCTATATCAT  
 GGCGACAAG CAGAAGAACG GCATCAAGGT GAACTTCAAG ATCCGCCACA  
 ACATCGAGGA CGGCAGCGTG CAGCTGCCG ACCACTACCA GCAGAACACC  
 CCCATCGCGC ACGGCCCCGT GCTGCTGCCG GACAACCACT ACCTGAGCAC  
 CCAGTCCGCC CTGAGCAAAG ACCCAACGA GAAGCGCGAT CACATGGTCC  
 TGCTGGAGTT CGTGACCGCC GCCGGGATCA CTCTCGGCAT GGACGAGCTG  
 TACAAGTAAA GCGGCCGCGA CTCTAGATCA TAATCAGCCA TACCACATT  
 GTAGAGGT TTACTTGCTT AAAAACCTC CCACACCTCC CCCTGAACCT  
 GAAACATAAA ATGAATGCAA TTGTTGTTGT TAAACTGTTT ATTGCAGCTT  
 ATAATGGTTA CAAATAAAGC AATAGCATCA CAAATTCAC AAATAAAGCA  
 TTTTTTCAC TGCATTCTAG TTGTTGTTG TCCAAACTCA TCAATGTATC  
 TTAAGCGTA AATTGTAAGC GTTAATATTT TGTTAAAATT CGCGTTAAAT  
 TTTTGTAAA TCAGCTCATT TTTAACCAA TAGGCCGAAA TCGGCAAAT  
 CCCTTATAAA TCAAAAGAAT AGACCGAGAT AGGGTTGAGT GTTGTCCAG  
 TTTGGAACAA GAGTCCACTA TTAAAGAACG TGGACTCCAA CGTCAAAGGG  
 CGAAAAACCG TCTATCAGGG CGATGGCCCA CTACGTGAAC CATCACCTA  
 ATCAAGTTT TTGGGGTCGA GGTGCCGTAA AGCACTAAAT CGGAACCCCTA  
 AAGGGAGCCC CCGATTAGA GCTTGACGGG GAAAGCCGGC GAACGTGGCG  
 AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG GGCGCTAGGG CGCTGGCAAG  
 TGTAGCGGTC ACGCTGCGCG TAACCACAC ACCCGCCGCG CTTAATGCGC  
 CGCTACAGGG CGCGTCAGGT GGCACTTTC GGGGAAATGT GCGCGGAACC  
 CCTATTGTT TATTTTCTA AATACATTCA AATATGTATC CGCTCATGAG  
 ACAATAACCC TGATAAAATGC TTCAATAATA TTGAAAAAAGG AAGAGTCCTG  
 AGGCGGAAAG AACCAGCTGT GGAATGTGTG TCAGTTAGGG TGTGGAAAGT  
 CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCATGCA TCTCAATTAG  
 TCAGCAACCA GGTGTGGAAA GTCCCCCAGGC TCCCCCAGCAG GCAGAAGTAT  
 GCAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCG CCCCTAACTC  
 CGCCCATCCC GCCCCTAAC CCGCCCAGTT CCGCCCATTC TCCGCCCAT  
 GGCTGACTAA TTTTTTTTAT TTATGCAGAG GCCGAGGCCG CCTCGGCCCTC  
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 GCAAAGATCG ATCAAGAGAC AGGATGAGGA TCGTTTCGCA TGATTGAACA  
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 ATCTCACCTT GCTCCTGCCG AGAAAGTATC CATCATGGCT GATGCAATGC  
 GCGGCTGCA TACGCTTGAT CCGGCTACCT GCCCATTGCA CCACCAAGCG  
 AAACATCGCA TCGAGCGAGC ACGTACTCGG ATGGAAGCCG GTCTTGTGCA  
 TCAGGATGAT CTGGACGAAG AGCATCAGGG GCTCGCGCCA GCCGAACTGT  
 TCGCCAGGCT CAAGGCGAGC ATGCCCGACG GCGAGGATCT CGTCGTGACC  
 CATGGCGATG CCTGCTTGCC GAATATCATG GTGGAAAATG GCCGCTTT  
 TGGATTCATC GACTGTGGCC GGCTGGGTGT GGCGGACCGC TATCAGGACA  
 TAGCGTTGGC TACCCGTGAT ATTGCTGAAG AGCTTGGCGG CGAATGGGCT

(cont. in Fig. 3C)

(cont. from Fig. 3B)

GACCGCTTCC TCGTGCTTTA CGGTATCGCC GCTCCCGATT CGCAGCGCAT  
CGCCTTCTAT CGCCTTCTTG ACGAGTTCTT CTGAGCGGGA CTCTGGGTT  
CGAAATGACC GACCAAGCGA CGCCCAACCT GCCATCACGA GATTTCGATT  
CCACCGCCGC CTTCTATGAA AGGTTGGGCT TCGGAATCGT TTTCCGGGAC  
GCCGGCTGGA TGATCCTCCA GCGCGGGGAT CTCATGCTGG AGTTCTTCGC  
CCACCCTAGG GGGAGGCTAA CTGAAACACG GAAGGAGACA ATACCGGAAG  
GAACCCGCGC TATGACGGCA ATAAAAAAGAC AGAATAAAAC GCACGGTGT  
GGGTCGTTTG TTCATAAACG CGGGGTTCGG TCCCAGGGCT GGCACCTCTGT  
CGATACCCCA CCGAGACCCC ATTGGGGCCA ATACGCCCGC GTTTCTTCCT  
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AACGTCGGGG CGGCAGGCC TGCCATAGCC TCAGGTTACT CATATATACT  
TTAGATTGAT TTAAAACCTTC ATTTTAATT TAAAAGGATC TAGGTGAAGA  
TCCTTTTGA TAATCTCATG ACCAAAATCC CTTAACGTGA GTTTCGTTTC  
CACTGAGCGT CAGACCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC  
TTTTTTCTG CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC  
CAGCGGTGGT TTGTTGCCG GATCAAGAGC TACCAACTCT TTTCCGAAG  
GTAACTGGCT TCAGCAGAGC GCAGATACCA AATACTGTCC TTCTAGTGT  
GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC  
TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG  
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GTCGGGCTGA ACGGGGGGTT CGTGCACACA GCCCAGCTTG GAGCGAACGA  
CCTACACCGA ACTGAGATAC CTACAGCGTG AGCTATGAGA AAGCGCCACG  
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AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC TGGTATCTT  
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TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT  
TTTACGGTTC CTGGCCTTT GCTGGCCTTT TGCTCACATG TTCTTCCTG  
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FIGURE 3C

## SEQUENCE LISTING

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|    | ctcagctaca aggaagctgt gcttcgtgct atagatggca tcaaccagcg gtcctcggat | 180 |
| 15 | gctaacctct accgcctcct ggacctggac cccaggccca cgatggatgg ggacccagac | 240 |
|    | acgccaaagc ctgtgagctt cacagtgaag gagacagtgt gccccaggac gacacagcag | 300 |
| 20 | tcaccagagg attgtgactt caagaaggac gggctggta agcggtgtat ggggacagtg  | 360 |
|    | accctcaacc aggccagggg ctcctttgac atcagttgtg ataaggataa caagagattt | 420 |
|    | gccctgctgg gtgatttctt ccggaaatct aaagagaaga ttggcaaaga gttaaaaga  | 480 |
| 25 | attgtccaga gaatcaagga tttttgcgg aatcttgtac ccaggacaga gtccta      | 536 |

30

35

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE 2004/000111

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/17, A61P 17/02, C07K 14/47, A61K 47/44 // C12N 5/08  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, A61P, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI DATA, EPO-INTERNAL, PAJ, MEDLINE, BIOSIS, CHEM.ABS.DATA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | WO 02095076 A2 (KABUSHIKI KAISHA TOYOTA CHUO KENKYUSHO), 28 November 2002 (28.11.2002), page 26, table 1; claims 1-7,17,22-23<br>--  | 1-5                   |
| X         | Immnuotechnology, Volume 1, 1995, James W. Lerrick et al, "Anti-microbial activity of human CAP18 peptides, "pages 65-72, page 69, paragraph 3.3<br>--   | 1-5                   |
| X         | Biophysical Journal, Volume 80, June 2001, Thomas Gutzmann et al, "Interaction of CAP18-Derived Peptides with Membranes Made from Endotoxins or Phospholipids", pages 2935-2945, table 1; page 2938, column 1, Results<br>-- | 1-5                   |

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

27 April 2004

Date of mailing of the international search report

30-04-2004

Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM

Authorized officer  
IDA CHRISTENSEN/BS

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE 2004/000111

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | Antimicrobial Agents and Chemotherapy, Volume 37, No. 12, 1993, James W. Lerrick et al, "Antimicrobial Activity of Rabbit CAD18-Derived Peptides", pages 2534-2539, page 2535, column 2, paragraph 1; page 2537, column 2, paragraph 3; page 2538, column 1, paragraph 5<br>-- | 1-5                   |
| X         | WO 9609322 A2 (CHILDREN'S MEDICAL CENTER CORPORATION), 28 March 1996 (28.03.1996), page 2, line 13 - line 33; page 9, line 11 - line 29; page 11, line 9 - line 17, page 18, lines 9-24  | 6-16,18-26            |
| A         | --   | 17                    |
| A         | --   | 1-26                  |
| X         | Antimicrobial Agents and Chemotherapy, Volume 45, No. 10, 2001, Lisa Saiman et al, "Cathelicidin Peptides Inhibit Multiply Antibiotic-Resistant Pathogens from Patients with Cystic Fibrosis", pages 2838-2844, page 2840, column 1, "assesment of epithelial toxicity"<br>--  | 21                    |
| A         | The Journal of Biological Chemistry, Volume 274, No. 32, 1999, Ole Sorensen et al, "The Human Antibacterial Cathelicidin, hCAP-18, Is Bound to Lipoproteins in Plasma", pages 22445-22451, page 22450, column 2, paragraphs 1-3<br>--  | 11-14,16,18, 21-23,25 |
| A         | The Journal of Biological Chemistry, Volume 273, No. 50, 1998, Yuqin Wang et al, "Apolipoprotein A-I Binds and Inhibits the Human Antibacterial/Cytotoxic Peptide LL-37", pages 33115-331118, abstract, page 33118<br>--   | 11-14,16,18, 21-23,25 |

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE 2004/000111

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.   |
|-----------|--|-------------------------|
| X         | WO 0057895 A1 (BETH ISRAEL DEACONESS MEDICAL CENTER), 5 October 2000 (05.10.2000), page 1, line 28 - line 32; page 26, line 30 - line 32; page 29, line 25 - line 29, page 31, line 15 - line 19   | 11-16,18,21             |
| A         | ---  | 1-10,17,<br>19-20,22-26 |
| A         | Wound Repair and Regeneration, Volume 9, No. 2, March-April 2001, Eleventh Annual Meeting and Educational Symposium Wound Healing Society; Albuquerque, New Mexico, USA; May 16-18, 2001, Pestonjamasp et al. "Antimicrobial peptides are effectors of wound repair", pg. 158<br>--<br>----- | 1-26                    |

**INTERNATIONAL SEARCH REPORT**

International application No.  
**PCT/SE2004/000111**

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
  - a. type of material
    - a sequence listing
    - table(s) related to the sequence listing
  - b. format of material
    - in written format
    - in computer readable form
  - c. time of filing/furnishing
    - contained in the international application as filed
    - filed together with the international application in computer readable form
    - furnished subsequently to this Authority for the purposes of search
2.  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

**INTERNATIONAL SEARCH REPORT**

International application No.  
**PCT/SE2004/000111**

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: **Claims: 24 (entirely), 26 (partially)**

**see extra sheet**

2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.  
**PCT/SE2004/000111**

**Box II.1**

Claim 24 relates to methods of treatment of the human or animal body by surgery or by therapy, and claim 26 may relate to methods of treatment of the human or animal body by surgery or by therapy (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds or compositions.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

31/03/2004

International application No.  
PCT/SE 2004/000111

|       |          |       |            |       |              |            |
|-------|----------|-------|------------|-------|--------------|------------|
| WO    | 02095076 | A2    | 28/11/2002 | JP    | 2003038192 A | 12/02/2003 |
|       |          |       |            | JP    | 2003250578 A | 09/09/2003 |
| ----- | -----    | ----- | -----      | ----- | -----        | -----      |
| WO    | 9609322  | A2    | 28/03/1996 | AU    | 3822895 A    | 09/04/1996 |
|       |          |       |            | US    | 5654273 A    | 05/08/1997 |
|       |          |       |            | US    | 5863897 A    | 26/01/1999 |
| ----- | -----    | ----- | -----      | ----- | -----        | -----      |
| EP    | 0935965  | A1    | 18/08/1999 | CA    | 2252467 A    | 30/10/1997 |
|       |          |       |            | WO    | 9739764 A    | 30/10/1997 |
| ----- | -----    | ----- | -----      | ----- | -----        | -----      |
| WO    | 0057895  | A1    | 05/10/2000 | AU    | 4013200 A    | 16/10/2000 |
|       |          |       |            | CA    | 2368638 A    | 05/10/2000 |
|       |          |       |            | EP    | 1165111 A    | 02/01/2002 |
|       |          |       |            | US    | 2004009463 A | 15/01/2004 |